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(54) Title: RECOMBINANT IGF BINDING PROTEIN (IBP-1)

(I)

A-P-W-Q-C-A-P-C-S-A-E-K-L-A-L-C-P-P-V-S-A-S-C-S-E-V-T-R-S-A-
G-C-G-C-C-P-M-C-A-L-P-L-G-A-A-C-G-V-A-T-A-R-C-A-R-G-L-S-C-R-
A-L-P-G-E-Q-Q-P-L-H-A-L-T-R-G-Q-G-A-C-V-Q-E-S-D-A-S-A-P-H-A-
A-E-A-G-S-P-E-S-P-E-S-T-E-I-T-E-E-E-L-L-D-N-F-H-L-M-A-P-S-E-
E-D-H-S-I-L-W-D-A-I-S-T-Y-D-G-S-K-A-L-H-V-T-N-I-K-K-W-E-P-C-
R-I-E-L-Y-R-V-V-E-S-L-A-K-A-Q-E-T-S-G-E-E-I-S-K-F-Y-L-P-N-C-
N-K-N-G-F-Y-H-S-R-Q-C-E-T-S-M-D-G-E-A-G-L-C-W-C-V-Y-P-W-N-G-
K-R-I-P-G-S-P-E-I-R-G-D-P-N-C-Q-M-Y-F-N-V-Q-N-

(57) Abstract

An IGF binding protein which has the amino acid sequence (I), or an equivalent modification thereof, such as a glycosylated modification. Further is indicated a DNA-sequence, coding for the protein, an expression vector and a pharmaceutical preparation containing the protein. The protein is effective as a potentiator for the function of IGF-compounds.

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Recombinant IGF binding protein (IBP-1)

The present invention relates to an Insulin-like Growth Factor Binding Protein, IBP-1, having a molecular weight of about 28 kD, derived from human placenta/endometrium, and equivalent modifications thereof.

The invention further relates to a DNA-structure, coding for the IBP-1, expression vectors containing this DNA-structure and procaryotic or eucaryotic cells comprising such a vector.

The invention still further relates to pharmaceutical preparations comprising IBP-1.

IGF or Insulin like Growth Factor is synonymous with somatomedins. The family of somatomedins are members of a group of polypeptides derived from the insulin gene. The gene products include insulin, insuline-like growth factor (IGF)I and II, relaxin and the B-unit of nerve growth factor (NGF) (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b; Bradshaw 1978; Isaacs et al., 1978). IGF elicits classical insulin effects on all target tissues of insulin, i.e. IGF increases glucose metabolism of adipose tissue and stimulates lipid, glycogen, and protein synthesis. IGF also stimulates DNA synthesis in certain cell types. This feature reflects the capacity of IGFs to induce cell proliferation and promote organ growth <u>in vivo</u>. Furthermore, IGF acts on differentiation of mesenchymal cells (Froesch et al., 1985).

IGF-I and IGF-II are unique in that they are complexed to specific binding proteins in plasma (Smith, 1984). At least two different binding proteins have been iden-

tified in adult human serum, namely (1) binding protein 53 (BP-53) a GH dependent binding protein, believed to be derived from the 150 kD complex which carries most of the endogenous IGF peptides, (2) IBP-1, an IGF binding protein of about 30-40 kD which is tissue specifically expressed in endometrium and liver and accounts for most of the unsaturable binding sites in plasma. While the 53 kD-binding protein is under GH control the 30-40 kD species appears to be expressed in a GH independent way.

The lower molecular weight binding protein was initially identified in human amniotic fluid and has been purified and characterized (Chochinov et al., 1977; Drop et al., 1979; Drop et al., 1982). This 30-40 kD IGF binding protein appears to be identical to binding proteins that have been purified from human serum and the human hepatoma cell line, HEPG2 (Drop et al., 1984a; Povoa et al., 1984; Povoa et al., 1985). Povoa et al showed that the NH_2-terminal amino acid sequence of the binding protein found in amniotic fluid and from the HEPG2 cell line are similar (Povoa et al., 1985).

Placental protein PP12, a protein originally isolated from human placenta, was found to bind IGF as well as to have an identical NH_2-terminal amino acid sequence (Koistinen et al., 1986).

As to the biological function of IGF-binding protein both stimulatory and inhibitory effects have been described.

Stimulatory effects of IGF-binding protein has been shown in at least two cases. Clemmons et al (1986) showed increased binding to fibroblast and smooth muscle cell surface receptors of IGF in complex with its binding protein.

Inhibitory effects of IGF-binding protein on various IGF actions in vitro, including stimulation of glucose transport by adipocytes, sulphate incorporation by chondrocytes and thymidine incorporation in fibroblasts have been described (Zapf et al., 1979; Drop et al., 1979; Ooi et al., 1986). In addition, inhibitory effects of IGF-binding proteins on growth factor mediated mitogen activity in normal cells (cartilage assay, Drop, thesis, 1983).

According to the invention, the IGF-binding protein has the following amino acid sequence:

Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-Glu-Lys-Leu-Ala-Leu-Cys-Pro-Pro-Val-Ser-Ala-Ser-Cys-Ser-Glu-Val-Thr-Arg-Ser-Ala-Gly-Cys-Gly-Cys-Cys-Pro-Met-Cys-Ala-Leu-Pro-Leu-Gly-Ala-Ala-Cys-Gly-Val-Ala-Thr-Ala-Arg-Cys-Ala-Arg-Gly-Leu-Ser-Cys-Arg-Ala-Leu-Pro-Gly-Glu-Gln-Gln-Pro-Leu-His-Ala-Leu-Thr-Arg-Gly-Gln-Gly-Ala-Cys-Val-Gln-Glu-Ser-Asp-Ala-Ser-Ala-Pro-His-Ala-Ala-Glu-Ala-Gly-Ser-Pro-Glu-Ser-Pro-Glu-Ser-Thr-Glu-Ile-Thr-Glu-Glu-Glu-Leu-Leu-Asp-Asn-Phe-His-Leu-Met-Ala-Pro-Ser-Glu-Glu-Asp-His-Ser-Ile-Leu-Trp-Asp-Ala-Ile-Ser-Thr-Tyr-Asp-Gly-Ser-Lys-Ala-Leu-His-Val-Thr-Asn-Ile-Lys-Lys-Trp-Lys-Glu-Pro-Cys-Arg-Ile-Glu-Leu-Tyr-Arg-Val-Val-Glu-Ser-Leu-Ala-Lys-Ala-Gln-Glu-Thr-Ser-Gly-Glu-Glu-Ile-Ser-Lys-Phe-Tyr-Leu-Pro-Asn-Cys-Asn-Lys-Asn-Gly-Phe-Tyr-His-Ser-Arg-Gln-Cys-Glu-Thr-Ser-Met-Asp-Gly-Glu-Ala-Gly-Leu-Cys-Trp-Cys-Val-Tyr-Phe-Trp-Asn-Gly-Lys-Arg-Ile-Pro-Gly-Ser-Pro-Glu-Ile-Arg-Gly-Asp-Pro-Asn-Cys-Gln-Met-Tyr-Phe-Asn-Val-Gln-Asn

The complete nucleotide sequence of the corresponding cDNA sequence was shown to have the structure depicted in claim 5.

The protein according to the invention may be used as an effective potentiator for the functioning of

somatomedins. The effect can be mediated through the firm binding between the somatomedins and their binding proteins under physiological conditions. Such complexed somatomedins together with their binding proteins are protected against undue proteolysis, causing a significant increase of the biological half life of somatomedins. Furthermore, this IGF-binding protein or modifications hereof might function as a potent carrier of IGF to its local sites of action.

The studies of Clemmons (1986) demonstrate the potential usefulness of this IGF-1 binding protein or modifications hereof in fertilization and in potentiation of growth of connective tissue and muscle cells in tissue repair.

As IGF-binding protein, or modifications thereof, such as alpha 1 PEG, are the major secretory soluble protein of decidual cells of the endometirum, IBP-1 may have an important function in restricting trophoblast invasion into the endometrium during placental development. Furthermore, the inhibitory function of IBP-1 in cellular proliferation assays and the unexpected direct inhibitory effect of IBP-1 on the oestrogen response on certain cancer cells make IBP-1 or modifications hereof a potential anticancer reagent with local growth inhibitory effect.

The invention is explained more in detail in the following description with reference to the drawing, in which

fig. 1 illustrates a restriction map and sequence strategy for human 28 kD IGF binding protein cDNA clones,

fig. 2 shows the nucleotide and deducted amino acid sequence of human IGF binding protein where differences between the placental cDNA sequence and the liver cDNA sequence are shown in parenthesis, and

fig. 3 represents an SDS/PAGE analysis of culture media of COS-1 cells transfected with pSV19, pSV4, pSV4Inv and untransfected COS-1 cells.

The cDNA encoding IGF-BP was obtained by screening a human placental and a human hepatoma (HEPG-2) cDNA-expression library with a polyclonal antibody to human amniotic fluid binding protein (Drop et al., 1984a).

Restriction analysis indicated that the clones isolated from the placenta library and the clones isolated from the HEPG2 library were colinear (fig. 1), supporting their candidacy as IGF binding protein clones. The composite restriction map is shown at the top of the figure 1. A putative leader sequence is shown in front of the open box representing translated regions. Each arrow shows the direction and extend of sequencing by chain termination. Four different clones are aligned. p4 and p19 originate from the placental cDNA library, while w61 and w85 originate from the HEPG2 cDNA library, (E = EcoRI, P = PstI, B = BamHI, H = HindII, S = SstI, X = XbaI, N = NcoI).

The complete nucleotide sequence of the cDNA insert of one of the clones isolated (p19) was determined. The 1421 nucleotide sequence shown in fig. 2 contains a 5' untranslated region of 52 nucleotides followed by an ATG codon and an open reading frame of 776 nucleotides. The potential initiation codon is flanked by 5 sequences matching Kozak's criteria for an initiation codon (Kozak, 1986). At the 3' end the open reading frame is flanked by a translation termination codon (TGA) and a 569 nucleotides long 3' untranslated sequence.

The open reading frame in cDNA clone p19 has a coding capacity for a protein of 259 residues also shown in

figure 2 (by the one-letter code), with a calculated Mw of 28,172 daltons. The initiation methione is the first amino acid of a 24-residue highly hydrophobic peptide sequence (underlined), representing the sequence of a putative signal sequence necessary for transfer of the nascent polypeptide sequence into the membranes of the endoplasmatic reticulim. A favourable signal peptidase cleavage site (ala-gly) occurs immediately N-terminally of the alanine residue at pos +1 (von Heijne, 1987). The NH_2-terminus of the predicted mature protein is identical to the chemically determined NH_2-terminus described for the IGF-binding protein isolated both from amniotic fluid (Povoa et al., 1984), and from the HEPG2 cell line (Povoa et al., 1985) and from serum (Baxter et al., 1987).

Omitting the signal peptide sequence, the M_r of this gene product is predicted to be 2,350 daltons. The M_r of serum IGF binding protein is about 28,000 daltons (Baxter et al., 1987). The difference is believed to be accounted for by glycosylation of the IGF binding protein (Bohn et al., 1980; Koistinen et al., 1986).

The amino acid sequence did not disclose N-linked glycosylation sites (N-T, N-S). However, at least five potential O-linked glycosylation sites were found in the NH_2-terminal of the molecule. A RGD sequence in the COOH terminal part of the IBP-1 protein has been found. Such a short sequence is considered to be important for cellular attachment of matrix proteins, such as fibronectin, vitronectin and von Willebrand factor, to receptors of the integrin family.

Amino acid homology with other known proteins and peptides were determined by searching the NFBR data base, version 12.0 and 26.0. The IGF-BP protein did not show

any significant protein homology, indicating that IGF-BP is a unique protein. In particular, comparison of amino acid sequence of IGF-I, IGF-II, Insulin, Type I IGF receptor and the type II IGF receptor amino acid sequences revealed no homologous domains. In addition, no homology was found with the reported NH_2-terminal amino acid sequence for the high molecular weight IGF binding protein (Baxter et al., 1986).

It was further possible to express IBP-1 in mammalian cells. Expression vectors, pSV19, pSV4 and pSV4Inv were constructed by inserting the full length clones p4 and p19 in the expression vector pSV328. The vectors pSV4, pSV19 and pSV4Inv, in which the cDNA insert is in 3' - 5' orientation, were transfected to COS-1 cells. By use of SDS/PAGE analysis cell culture media from COS-1 cells transfected with pSV19 (lane A), pSV4 (lane B), and amniotic fluid (lane C) were analysed. The IGF binding proteins were made visible by immuno staining as described for the screening of the cDNA libraries. In culture media of pSV4 and pSV19 transfected COS-1 cells in which the gene is in the correct orientation a protein of 32 kD being immunologically indistinguishable from the IGF binding protein from amniotic fluid (fig. 3) was detected. In all culture media a band was visible which reacted with the 35 kD SMBP antibody but which was absent in the culture medium from untransfected COS-1 cells.

Although IBP-1 successfully has been expressed in COS-1 cells the lack of N-linked glycosylation sites in the putative protein also favour expression in yeast and bacteria to increase the IBP-1 production to be used in a variety of therapeutic compositions.

The invention provides therapeutic compositions comprising IBP-1 or derivatives thereof and pharmacologically

acceptable excipients. Such compositions including the IGF-binding protein or derivatives hereof according to this invention have many therapeutic uses involving the physiological functions of somatomedins.

The IGF-binding protein of the invention may be formulated as pharmaceutical preparations comprising the IGF-binding protein of the invention together with the usual excipients. Pharmaceutical preparations according to the invention may be in the form of suspension or solutions for parentheral administration, e.g. i.v., s.c., i.m., implants, subcutaneous or interveneous administration or administration through the mucosa, e.g. oral, nasal, buccal, sublingual or rectal administration or transdermal administration.

For example in cases where somatomedins have to be transported to specific target tissues in a way where the physiological halflife of the somatomedins has to be increased by complexing IBP-1 described in this invention to IGF-1 and IGF-2. In accordance with this invention a slow release of active IGF-1 or 2 from such complexes would ascertain a constant level of somatomedins either locally or systemically dependent upon the way of administration. IBP-1 describes in this invention hereby abolishes the potent mitogenic effect of the somatomedins that administrated in high dosis, i.e. intra venously, would cause unwanted local cellular proliferations in a variety of cells like fibroblasts, muscle cells and endothelial cells.

However, the IBP-1 described by this invention administred together with IGF-1, IGF-2 and other growth factors or formulated as common preparations for topical use (such as PDGF, EGF, FGF, TGFalpha or TGFbetha) employed in therapeutical devices to be used in healing of wounds or in treatment of oeteoporosis and in healing

of bones might be valuable for a steady and controlled release of the somatomedins in such therapeutical devices.

Such preparations may optionally be administred in the form of combination preparations e.g. comprising IBP-1 and IGF-1, IBP-1 and IGF-2 or IBP-1, IGF-1 and IGF-2.

In general, IBP-1 according to this invention might turn out to exhibit a potent regulatory function in the release of IGF-1 and/or IGF-2 in future treatment of injuries or other malfunctions that requires increased IGF-1 and/or IGF-1 levels.

On the other hand IBP-1 or derivatives thereof according to this invention might be useful in therapy of the proliferation of certain cancers characterized by producing somatomedins in high amounts thus inhibiting the autocrine/paracrine physiological stimulation of unwanted cellular proliferation in cancers like chondrosarcomas, fibrosarcomas, and mammacarcinomas.

Furthermore, the IBP-1 or derivatives hereof described in this invention is useful for the production of antibodies. Such mono- or polyclonal antibodies are suitable for developing immunological methods like immunohistochemical analysis of IBP-1 in tissues and for developing ELISA for IBP-1 quantitation. Such ELISA will prove valuable for early screening the levels of IBP-1 in patients with altered IGF-1 and 2 levels.

Pharmaceutical preparations of this invention for s.c. and i.m. administration can be prepared by mixing the following constituents: IBP-1 and derivatives thereof together with IGF-1, IGF-2 and other growth factors, an isotonic agent, a buffer, a preservative and water.

After mixing the pH value of the preparation is, if necessary, adjusted to pH = 7.3.

Examples of preservatives: phenol and m-cresol. Examples of an isotonic agent: sodium chloride and glycerol. Example of buffer is sodium phosphate.

Pharmaceutical preparations of this invention for transmucosal administration can be prepared by mixing the following constituents: IBP-1 and derivatives thereof, together with IGF-1, IGF-2 and other growth factors, a buffer, an isotonic agent, a preservative, an absorption promotor and a vehicle e.g. water, cellulose, water-soluble cellulose alkylethers, crystalline cellulose, water-soluble polyacrylates or mixtures thereof.

Pharmaceutical preparations of this invention for transdermal administration can be prepared by mixing the following constituents: IBP-1 and derivatives thereof together with IGF-1, IGF-2 and other growth factors, an isotonic agent, a preservative and a vehicle e.g. a hydrophilic gel of water-soluble cellulose alkylethers.

This invention is further explained in the following working example describing the isolation and characterization of IBP-1.

EXAMPLE

Screening the Lambda gtll Expression Library

A human placenta cDNA library in lambda gtll and a cDNA library of the human hepatoma cell line HEPG2 were screened with a polyclonal antibody to human amniotic fluid binding protein according to the procedure described by Young and Davis (Young and Davis, 1982).

Rabbit antibody to 35 kD somatomedin binding protein SMBP isolated from human amniotic fluid was produced and purified as described by Drop et al., 1984a. The antibody was absorbed against E.coli Y1090 and lambda gtll proteins by incubating with nitrocellulose filters that had been lifted from confluent lysis plates of E.coli Y1090/lambda gtll induced with 10 mM isopropyl beta-d-thiogalacopyranoside (IPTG). The antibody was further absorbed against human serum albumin immobilized on nitrocellulose filters. Approximately 4 x 10^5 clones of the placental library were screened and about 0.5 x 10^5 of the HPEG2 library. 3-5 x 10^4 plaque forming units per 150 mm Petri dish were plated on a lawn of Y1090 bacteria and incubated. After 2 hr incubation the plates were covered with nitrocellulose filters (Millipore HATF) that had been saturated with 10 mM IPTG. The plates were incubated at 37°C for 2-2.5 hrs. The filters were removed, washed with Tris-buffered saline (TBS; 10 mM Tris/HCl, pH 7.5/150 mM NaCl) at room temperature and incubated with 3% BSA in TBS for 30 min. at room temperature. Partly purified rabbit polyclonal 35 kD SMBP antibody diluted 1:125 was added to 3% BSA in TBS plus 0.02% azide, and the filters were incubated overnight at 4°C. The filters were washed and incubated for 60 min. at room temperature with horse-radish peroxydase conjugated goat anti-rabbit IgG (Tago) diluted 1:200 in 3% BSA in TBS. The filters were washed and stained with amidophenyl and napthol AS-MX phosphate in 0.2 M Tris/HCl, pH 9.2, 10 mM $MgCl_2$ at room temperature.

Positive phages were isolated and DNA was isolated by standard methods (Maniatis et al., 1982). About 33 plaques strongly cross-reacting with the polyclonal antibody were identified in the placenta and HEPG2 cDNA library. Following re-screening inserts varying in size between 0.9-1.5 Kb were isolated and subcloned

in the vector PTZ19 from Pharmacia. All isolated clones showed cross-hybridization in a Southern blot except one clone from the placenta library and the 5 weakly hybridizing clones from the HEPG2 library.

DNA was digested with various restriction endonucleases (BRL, NEN, Boehringer) according to the suppliers directions, electrophoresed in 0.8% agarose, and transferred to nitrocellulose filters according to the method of southern (Southern, 1975). mRNA was denaturated with dimethylsulfozide (DMSO) and glyoxal, subjected to electrophoresis in 1% agarose and transferred to nitrocellulose filters (Millipore HFTF).

Restriction fragments were subcloned in the vectors PTZ18 or PTZ19 (Pharmacia) and sequenced according to the chain termination method (Sanger et al., 1977). In regions which lacked convenient restriction sites, appropriate clones were generated by Bal 31 nuclease digestion.

Transfection of COS-1 Cells

The full length cDNA clones p4 and p19 were subcloned in the EcoR1 site of pSV328, which expressed cloned inserts using the simian virus 40 (SV40) early promotor (Van Heuvel et al., 1986). A DEAE-dextran procedure (McCuthchan & Pagano, 1986) followed by treatment with 100 uM chloroquine in Dulbecco's MEM (DMEM) for 4 hrs was used to transfect COS-1 cells (Gluzman, 1981). After this treatment the cells were grown 24 hrs with DMEM plus 5% foetal calf serum. Medium was removed after 72 hrs, and the cells were washed extensively with DMEM and incubated for 72 hrs with DMEM without serum. Production of 32 kD binding protein in culture media was determined using 35 kD SMBP antibody.

Purification of IBP-1

Proteins from amniotic fluid or from conditioned media were precipitated with ammonium sulphate at a final concentration of 35%. Following centrifugation the supernatant was brought to 50% ammonium sulphate. The pellet was dissolved in 45% ammonium sulphate and the final pellet was dissolved in 50 mM Tris HCl, pH 7.5 for further purification and characterization. The dissolved ammonium sulphate precipitate was further purified by reverse phase chromatography on C_{18}. Following washings with 50 mM Tris-HCl, pH 7.5 and Tris HCl, pH 7.5 in 50% methanol the pure IBP-1 was eluted from the column with Tris-HCl, pH 7.5 in 65% methanol. The IBP-1 was precipitated overnight in a 7% Trichloroacetic-acid solution. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.5, lyophilized and storred at ÷ 20°C.

Characterization of purified IBP-1

IBP-1 binds both IGF-1 and IGF-2 with approximately the same specificity when measured in binding assays with $|^{125}I|$IGF-1 and $|^{125}I|$IGF-2 with or without excess of cold iGF-1 or IGF-2. The specificity of IBP-1 reaction was tested in similar assays employing such competition assays and in assays in which IBP-1 - IGF binding was visualized through the specific reaction of antibody against IBP-1. In such assays IBP-1 did only react with IGF-1 and IGF-2 but not with closely related compounds like insulin, proinsulin or truncated forms thereof.

The biological effects of purified samples if IBP-1 were tested in in vitro mitogenicity tests employing ^{3}H-thymidin incorporation into a MCF-7 mamma carcinoma cell line. The stimulatory effect of both IGF-1 and

IGF-2 on cell proliferation was inhibited by IBP-1 in a dose dependent way. The effect of IBP-1 on IGF-2 dependent cell proliferation was more pronounced than that of the effect of IBF-1.

Furthermore, in an assay employing $|^{35}S|$-Methionin incorporation into cartillage the stimulatory effect of IGF-1 and IGF-2 was abolished by IBP in the low ng range.

Examples of pharmaceutical preparations:

EXAMPLE 1

25 ng IBP-1
10 ng IGF-1
0.7% NaCl
1/75M sodiumphosphate
water ad 1 ml

The calculated amounts of IBP-1 and IGF-1 were dissolved and diluted in phosphate buffer containing NaCl. The pH was adjusted to 7.3-7.4.

EXAMPLE 2

25 ng IBP-1
10 ng IGF-2
1.6% glycerin
1/75M sodiumphosphate
0.01% benzalconiumchlorid
0.05% sodiumedetat
water ad 1 ml

The calculated amounts of IBP-1 and IGF-2 were dissolved and diluted in phosphate buffer containing glycerin, benzalconiumchlorid and sodiumedetat. The pH

was adjusted to 7.4

EXAMPLE 3

25 ng IBP-1
10 ng IGF-1
10 ng IGF-2
5% hydroxyethylcellulose
0.9% benzylalcohol
1/75M phosphate buffer
water ad 1 ml

The gel is prepared by mixing hydroxyethylcellulose with the waterphase containing IBP-1, IGF-1 and IGF-2.

Patent Claims:

1. An IGF binding protein, comprising the following amino acid sequence:

A-P-W-Q-C-A-P-C-S-A-E-K-L-A-L-C-P-P-V-S-A-S-C-S-E-V-T-R-S-A-
G-C-G-C-C-P-M-C-A-L-P-L-G-A-A-C-G-V-A-T-A-R-C-A-R-G-L-S-C-R-
A-L-P-G-E-Q-Q-P-L-H-A-L-T-R-G-Q-G-A-C-V-Q-E-S-D-A-S-A-P-H-A-
A-E-A-G-S-P-E-S-P-E-S-T-E-I-T-E-E-E-L-L-D-N-F-H-L-M-A-P-S-E-
E-D-H-S-I-L-W-D-A-I-S-T-Y-D-G-S-K-A-L-H-V-T-N-I-K-K-W-E-P-C-
R-I-E-L-Y-R-V-V-E-S-L-A-K-A-Q-E-T-S-G-E-E-I-S-K-F-Y-L-P-N-C-
N-K-N-G-F-Y-H-S-R-Q-C-E-T-S-M-D-G-E-A-G-L-C-W-C-V-Y-P-W-N-G-
K-R-I-P-G-S-P-E-I-R-G-D-P-N-C-Q-M-Y-F-N-V-Q-N-

or an equivalent modification thereof.

2. A glycosylated modification of the protein according to claim 1.

3. An IGF binding protein according to claim 2, in which one or more hydroxy groups are glycosylated.

4. A DNA sequence, coding for the IGF binding protein as defined in claim 1.

5. A cDNA sequence according to claim 4 in which the coding strand includes the following structure:

```
   1  GGGCGGGCAC AGCCAGAGAG CATCGGCCCC TGTCTGCTGC TCGCGCCTGG
  51  AGATGTCAGA GGTCCCCGTT GCTCGCGTCT GGCTGGTACT GCTCCTGCTG
 101  ACTGTCCAGG TCGGCGTGAC AGCCGGCGCT CCGTGGCAGT GCGCGCCCTG
 151  CTCCGCCGAG AAGCTCGCGC TCTGCCCGCC GGTGTCCGCC TCGTGCTCGG
 201  AGGTCACCCG GTCCGCCGGC TGCGGCTGTT GCCCGATGTG CGCCCTGCCT
 251  CTGGGCGCCG CGTGCGGCGT GGCGACTGCA CGCTGCGCCC GGGGACTCAG
 301  TTGCCGCGCG CTGCCGGGGG AGCAGCAACC TCTGCACGCC CTCACCCGCG
 351  GCCAAGGCGC CTGCGTGCAG GAGTCTGACG CCTCCGCTCC CCATGCTGCA
 401  GAGGCAGGGA GCCCTGAAAG CCCAGAGAGC ACGGAGATAA CTGAGGAGGA
 451  GCTCCTGGAT AATTTCCATC TGATGGCCCC TTCTGAAGAG GATCATTCCA
 501  TCCTTTGGGA CGCCATCAGT ACCTATGATG GCTCGAAGGC TCTCCATGTC
 551  ACCAACATCA AAAAATGGAA GGAGCCCTGC CGAATAGAAC TCTACAGAGT
 601  CGTAGAGAGT TTAGCCAAGG CACAGGAGAC ATCAGGAGAA GAAATTTCCA
 651  AATTTTACCT GCCAAACTGC AACAAGAATG GATTTTATCA CAGCAGACAG
 701  TGTGAGACAT CCATGGATGG AGAGGCGGGA CTCTGCTGGT GCGTCTACCC
 751  TTGGAATGGG AAGAGGATCC CTGGGTCTCC AGAGATCAGG GGAGACCCCA
 801  ACTGCCAGAT GTATTTTAAT GTACAAAACT GAAACCAGAT GAAATAATGT
 851  TCTGTCACGT GAAATATTTA AGTATATAGT ATATTTATAC TCTAGAACAT
 901  GCACATTTAT ATATATGTAT ATGTATATAT ATATAGTAAC TACTTCTTAT
 951  ACTCCATACA TAACTTGATA TAGAAAGCTG TTTATTTATT CACTGTAAGT
1001  TTATTTTTTC TACACAGTAA AAACTTGTAC TATGTTAATA ACTTGTCCTA
1051  TGTCAATTTG TATATCATGA AACACTTCTC ATCATATTGT ATGTAAGTAA
1101  TTGCATTTCT GCTCTTCCAA AGCTCCTGCG TCTGTTTTTA AAGAGCATGG
1151  AAAAATACTG CCTAGAAAAT GCAAAATGAA ATAAGAGAGA GTAGTTTTTC
1201  AGCTAGTTTG AAGGAGGACG GTTAACTTGT ATATTCCACC ATTCACATTT
1251  GATGTACATG TGTAGGGAAA GTTAAAAGTG TTGATTACAT AATCAAAGCT
1301  ACCTGTGGTG ATAGTTGCCA CCTGTTAAAA TGTACACTGG ATATGTTGTT
1351  AAACACGTGT CTATAATGGA AACATTTACA ATAAATATTC TGCATGGAAA
1401  AAAAAAAAAA AAAAAAAAAA AAAAA
```

6. An expression vector, containing the DNA sequence defined in claim 4 or 5.

7. The expression vector pSV19.

8. The expression vector pSV4.

9. A cell line or a microorganism, comprising an expression vector in accordance with claims 6, 7 or 8.

10. A pharmaceutical preparation, comprising the binding protein defined in any of the claims 1-3.

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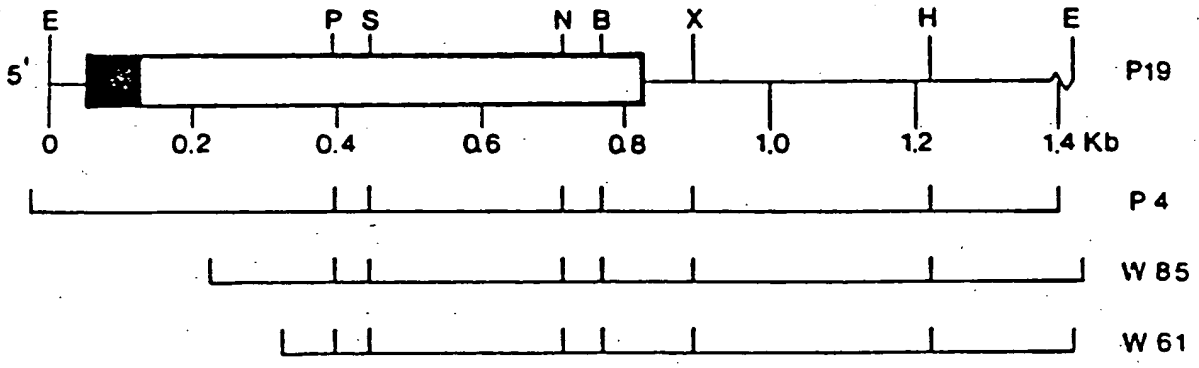
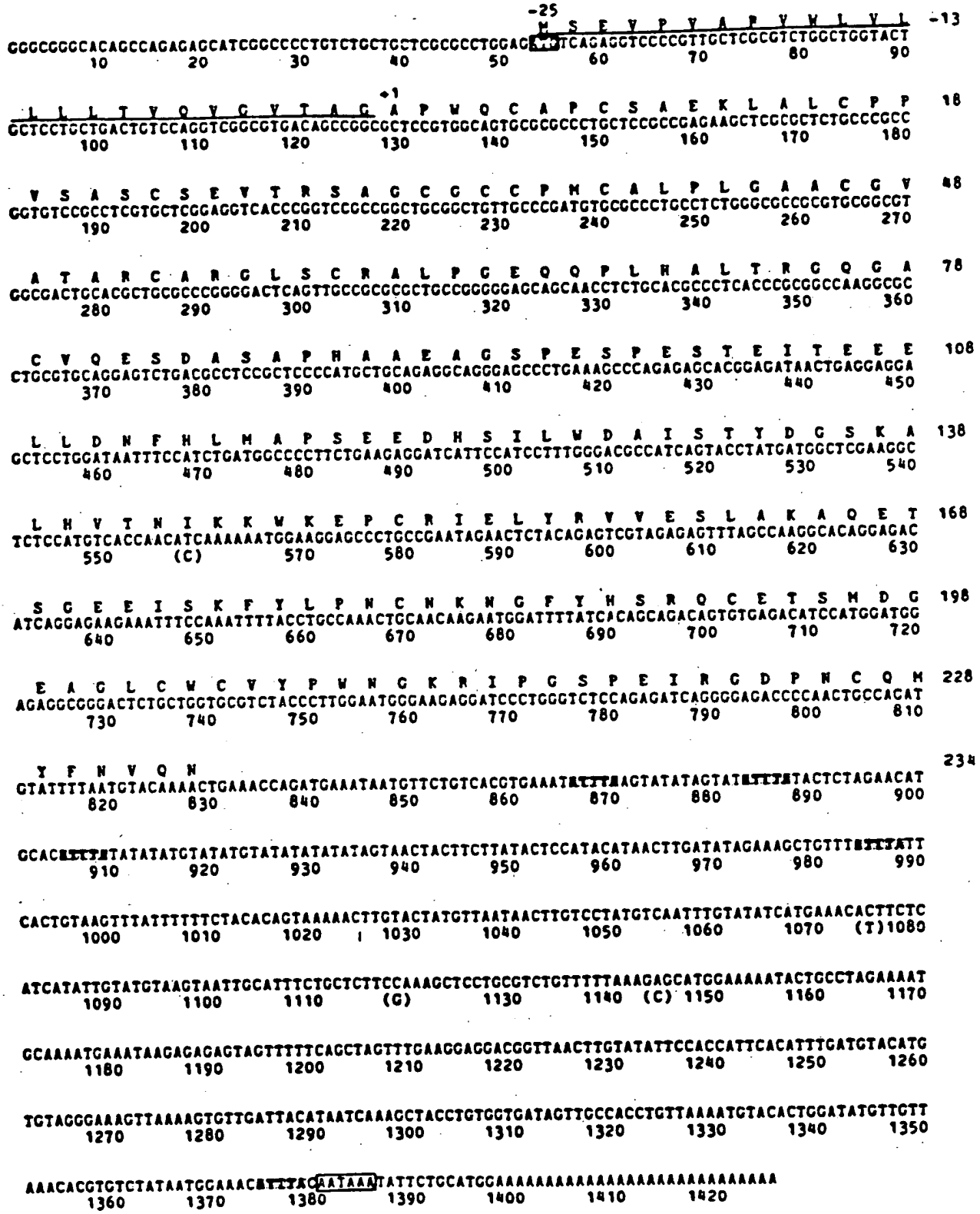


FIG.1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10 20 30 40 50 60 70 80 90
+1
L L L T V Q V G V T A G A P W Q C A P C S A E K L A L C P P 18
GCTCCTGCTGACTGTCCAGGTCGGCGTGACAGCCGGCGCTCCGTGGCAGTGCGCGCCCTGCTCCCCCGAGAAGCTCGCGCTCTGCCCGCC
100 110 120 130 140 150 160 170 180
V S A S C S E V T R S A G C G C C P M C A L P L G A A C G V 48
GGTGTCCGCCTCGTGCTCGGAGGTCACCCGGTCCGCCGGCTGCGGCTGTTGCCCGATGTGCGCCCTGCCTCTGGGCGCCCCGTGCGGCGT
190 200 210 220 230 240 250 260 270
A T A R C A R G L S C R A L P G E Q Q P L H A L T R G Q G A 78
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280 290 300 310 320 330 340 350 360
C V Q E S D A S A P H A A E A G S P E S P E S T E I T E E E 108
CTGCGTGCAGGAGTCTGACGCCTCCGCTCCCCATGCTGCAGAGGCAGGGAGCCCTGAAAGCCCAGAGAGCACGGAGATAACTGAGGAGGA
370 380 390 400 410 420 430 440 450
L L D N F H L M A P S E E D H S I L W D A I S T Y D G S K A 138
GCTCCTGGATAATTTCCATCTGATGGCCCCTTCTGAAGAGGATCATTCCATCCTTTGGGACGCCATCAGTACCTATGATGGCTCGAAGGC
460 470 480 490 500 510 520 530 540
L H V T N I K K W K E P C R I E L Y R V V E S L A K A Q E T 168
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550 (C) 570 580 590 600 610 620 630
S G E E I S K F Y L P N C N K N G F Y H S R Q C E T S M D G 198
ATCAGGAGAAGAAATTTCCAAATTTTACCTGCCAAACTGCAACAAGAATGGATTTTATCACAGCAGACAGTGTGAGACATCCATGGATGG
640 650 660 670 680 690 700 710 720
E A G L C W C V Y P W N G K R I P G S P E I R G D P N C Q M 228
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730 740 750 760 770 780 790 800 810
234
Y F N V Q N
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FIG. 2

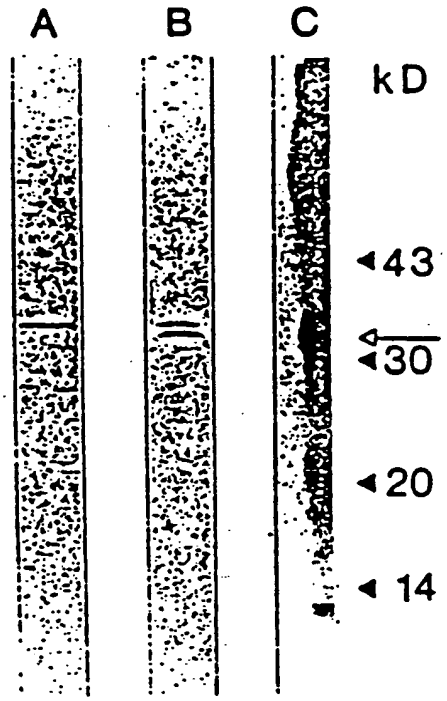

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 89/00008

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 07 K 13/00, C 12 N 15/00, C 12 P 21/02, A 61 K 37/02, //A 61 K 35/50

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC[4]	C 07 K; C 12 N; C 12 P; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP, A, 0141326 (BEHRINGWERKE) 15 May 1985, see the whole document --	1-3,10
X	Biochemical and Biophysical Research Communications, vol. 141, no. 1, 23 November 1986, Academic Press, Inc. (US) M.L. Huhtala et al.: "Biologically active domain in somatomedin-binding protein", pages 263-270 see the whole article --	1-3,10
P,X	Molecular Endocrinology, vol. 2, no. 5, May 1988 The Endrocrine Society Y.L. Lee et al.: "Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid from human HEP G2 hepatoma cells: predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors" pages 404-411 see the whole article --	1-10

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* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th June 1989	11. 07. 89
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document with indication where appropriate, of the relevant passages	Relevant to Claim No
P,X	Biochemical and Biophysical Research, vol. 152, no. 3, 16 May 1988 Academic Press, Inc. M.T. Brewer et al.: "Cloning characterization, and expression of a human insulin-like growth factor binding protein", pages 1289-1297 see the whole article	1-10
P,X	Embo Journal, vol. 7, no. 8, 1988 IRL Press Ltd. Oxford (GB) A. Brinkman et al.: "Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1) pages 2417-2423 see the whole article	1-10
P,X	FEBS-Lett., vol. 236, no. 2, August 1988, Elsevier Science Publishers B.V. Biomedical Division M. Julkunen et al.: "Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA", pages 295-302 see the whole article	1-10
P,X	Nucleic Acids Research, vol. 16, no. 17, 1988, IRL Press Ltd. Oxford (GB) U. Grundmann et al.: "Cloning of cDNA encoding human placental protein 12 (PP12):binding protein for IGF I and somatomedin" page 8711 see the whole article	1-10

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 8900008 SA 27516

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/07/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0141326	15-05-85	DE-A- 3338480	02-05-85
		AU-B- 569566	04-02-88
		AU-A- 3452784	02-05-85
		CA-A- 1234100	15-03-88
		DE-A- 3473279	15-09-88
		JP-A- 60104015	08-06-85
		US-A- 4592863	03-06-86